

Microbial competition for nitrogen and carbon is as intense in the subsoil as in the topsoil

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1 **Microbial competition for nitrogen and carbon is as intense in the subsoil as in the**
2 **topsoil**

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18 **ABSTRACT**

19 Most studies on plant nutrition tend to focus on the topsoil (plough layer) and frequently
20 neglect subsoil processes. However, cereal roots can potentially acquire nutrients
21 including organic and inorganic nitrogen (N) from deep in the soil profile. Greater
22 knowledge on the interaction of plants and microbes in subsoil environments is required
23 to evaluate whether deep rooting traits in cereals will achieve greater nutrient use
24 efficiency and greater soil carbon (C) storage in cropping systems. This study aimed to
25 evaluate the relationship between root distribution, organic and inorganic N availability
26 and potential N supply at the critical growth period during the wheat cropping cycle in
27 a sand textured Eutric Cambisol. Our results provide evidence of significant microbial
28 capacity in the subsoil. The rate of plant residue turnover and the mineralization of
29 organic C and N substrates (glucose, amino acids, peptides, protein) declined slightly
30 with increasing soil depth; however, these rates were not correlated with basal soil
31 respiration, microbial biomass or community structure. This suggests that the microbial
32 population in subsoil is more C limited but that its activity can be readily stimulated
33 upon C substrate addition. A significant potential for organic and inorganic N turnover
34 was also demonstrated at depth with a similar abundance of ammonifiers and ammonia
35 oxidizing bacteria (AOB) and archaea (AOA) throughout the soil profile. Again, N
36 mineralization in subsoils appears to be substrate limited. Root density declined rapidly
37 down the soil profile with few roots present past 50 cm; suggesting that this is the major
38 factor limiting C recharge of soil organic matter and microbial activity in subsoils.
39 Greater root proliferation at depth could allow greater capture of water and the recapture
40 of N lost by leaching; however, our results suggest that plant-microbial competition for
41 C and N is as intense in the subsoil as in the topsoil. We conclude that while deeper
42 rooting may improve nutrient and water use efficiency it may not lead to much greater
43 C sequestration in subsoils, at least in the short term.

44

45 *Keywords:* Ammonium; Dissolved organic nitrogen; Nitrate; Nitrification; Rhizosphere

46

47 **1. Introduction**

48 In high input agricultural systems, nitrogen (N) availability is largely controlled
49 by fertilizer events and the subsequent transformation and redistribution of N within the
50 soil (Van Egmond et al., 2002). Typically, however, only 50% of the N applied to the
51 crop in temperate climates is taken up by the plant indicating low rates of N use
52 efficiency (Lassaletta et al., 2014). In many countries, however, there is a move to
53 reduce the reliance on mineral fertilizers and to use added and intrinsic soil N reserves
54 more efficiently (Chen et al., 2016). Ultimately, this aims to reduce economic costs as
55 well as simultaneously lowering losses via leaching (NO_3^-), denitrification ($\text{N}_2/\text{N}_2\text{O}$)
56 and volatilization (NH_3). Increases in N efficiency can potentially be achieved using a
57 range of plant-based strategies (e.g. changes in root architecture combined with deeper
58 rooting, release of nitrification inhibitors, use of N_2 -fixers; Liu et al., 2013) as well as
59 changes in agronomic practice (e.g. improvements in fertilizer timing, formulation,
60 placement; Hoyle and Murphy, 2011; Sartain and Obrezai, 2010). Under some of these
61 scenarios it is likely that plants will have to take up and utilise a wider range of organic
62 and inorganic N forms (e.g. amino acids, peptides and polyamines). We hypothesize
63 that this will increase the competition between plant roots and soil microbial community
64 associated with both the mineralization of N contained in soil organic matter (SOM)
65 (via the direct release of root proteases or stimulation of SOM priming) and the capture
66 of any N released in both the topsoil and subsoil (Bardgett et al., 2003; Farrell et al.,
67 2013; Kaiser et al., 2015).

68 As soils frequently become progressively drier during the growing season,
69 there is a decreased root capture of water and nutrients from the topsoil, leading to the

70 growth of a few roots to depths often in excess of 1 m (DuPont et al., 2014). This
71 suggests that the subsoil may play a more significant role in N supply later in the
72 season, especially under reduced fertilizer input regimes. This may also promote
73 carbon (C) sequestration in subsoils although the evidence to support this remains
74 controversial (Agostini et al., 2015; Menichetti et al., 2015). Plant and microbial N
75 cycling in deeper soil horizons, however, have received much less attention than in
76 surface soils. If we are to capitalize on the deep rooting phenomenon of most cereals
77 and the potential to manipulate root architecture (breeding, genetic modification; Fang
78 et al., 2017), it is important that we understand water and nutrient availability in deeper
79 soil layers as well as the microbial processes that control them (e.g. SOM dynamics;
80 Zhang et al., 2014).

81 Agronomic estimates of N supply to plants are typically predicted from the
82 amount of inorganic N released during the laboratory incubation of soils collected
83 within the plough layer (0-30 cm). These mineralization rates are unlikely to be
84 representative of deeper soil layers and ways of integrating potential N supply from
85 subsoil is therefore needed. The amount and turnover of N in subsoil will largely depend
86 on its exchange capacity, structure, organic material availability and microbial activity.
87 It is well established that significant microbial activity may occur at depth (Doran, 1987;
88 Soudi et al., 1990), albeit at much lower levels and with a different community structure
89 than occurs in topsoil (Federle et al., 1986). When considering microbial processes at
90 depth a key component with respect to N cycling is the abundance of ammonia-oxidising
91 archaea (AOA) and bacteria (AOB) that are responsible for the rate limiting step in
92 nitrification and thus potential N loss. Dominance of AOA relative to AOB in the *amoA*
93 (ammonia monooxygenase) soil gene pool has been reported in many ecosystems
94 globally. Substrate availability and pH have been identified as the major drivers of niche
95 specialization between AOA and AOB, with AOA being reported to be more

96 competitive in acidic, organic matter depleted soil conditions at depth than AOB (He et
97 al., 2012; Zhang et al., 2012; Banning et al., 2015). However, variation in soil factors
98 such as water and oxygen availability are also important factors which differ in subsoil
99 and which may play a role in regulating population abundances to depth. The quantity
100 and quality of organic inputs to subsoil may also be different to the soil surface due to
101 lower rates of root and microbial turnover and the lack of leaf litter and crop residue
102 inputs. Subsoil soil organic matter has also been suggested to be older and more
103 recalcitrant than in the topsoil (Schrumpf et al., 2013; Torres-Sallan et al., 2017). While
104 this may favour C sequestration, it may conversely limit N supply to the plant.

105 Root length density (RLD) has been used as a proxy to predict water and nutrient
106 uptake by plants (Taylor and Klepper, 1975; Herkelrath et al., 1977). This relationship
107 can work well when there is adequate soil moisture available; however, it lacks precision
108 when surface soils become prone to drying. The root systems of mature wheat plants
109 typically extend deeper than 120 cm by the end of the growing season. However, the
110 time at which maximal crop N demand and subsoil exploitation coincide is earlier in the
111 season (i.e. Growth Stages GS31-71, stem elongation to the start of flowering; AHDB,
112 2015). Further, even though roots may extend deeper into the subsoil, their density may
113 be extremely low (Li et al., 2017). This study therefore aimed to evaluate the
114 relationship between root distribution, organic and inorganic N availability and potential
115 N supply at this critical period during a wheat growing cycle. We hypothesized that the
116 subsoil microbial population would be very low due to the lack of supply of available C
117 and N from plant roots and associated mycorrhizas. Further, this nutrient limitation
118 would lead to a more fungal and Gram+ dominated community and that this would be
119 slow to respond to C substrate addition leading to a greater potential to retain C in
120 subsoils. We also hypothesized that slow rates of organic N addition would lead to low

121 populations of AOA and AOB and little potential to generate NO_3^- , thus also favouring
122 N retention in subsoils.

123

124 **2. Materials and methods**

125 *2.1. Site characteristics*

126 Soil was collected from a replicated wheat field trial site located in
127 Abergwyngregyn, North Wales ($53^\circ 14' 29''\text{N}$, $4^\circ 01' 15''\text{W}$) and is classified as a sand
128 textured Eutric Cambisol. The soil pH is 6.3 and does not vary significantly with depth
129 (0-60 cm; $P > 0.05$). The bulk density in the topsoil (0-30 cm) is $1.48 \pm 0.12 \text{ g cm}^{-3}$ and
130 in the subsoil (30-60 cm) $1.63 \pm 0.10 \text{ g cm}^{-3}$. The climate at the site is classed as
131 temperate-oceanic with a mean annual soil temperature of 11°C at 10 cm depth and a
132 mean annual rainfall of 1250 mm yr^{-1} . The field trial consisted of six replicated plots
133 ($12.5 \times 3 \text{ m}$) which were ploughed (0-30 cm) and planted with spring wheat (*Triticum*
134 *aestivum* L. cv. Granary) in May 2013. Fertilizer was added after crop emergence (60
135 kg N ha^{-1} as ammonium nitrate, 80 kg K ha^{-1} , 28 kg P ha^{-1}) and dicot herbicides applied
136 following standard agronomic practice.

137 Soil water content, crop height and biomass were determined weekly by
138 destructive sampling throughout the growing season. Briefly, in six replicate plots, all
139 the crop biomass was removed within a sub-plot ($0.5 \text{ m} \times 0.5 \text{ m}$), the samples placed in
140 paper bags and the harvested biomass dried at 80°C for 7 d to determine dry weight. At
141 the same time, crop height was recorded at 5 points (1 m apart) within each of the six
142 plots. Soil water content was determined weekly by destructive sampling throughout the
143 growing season. Briefly, topsoil (0-30 cm) and subsoil (30-60 cm) samples were taken
144 from six replicate plots, sieved to pass 2 mm and a subsample used to determine
145 moisture content by drying at 105°C overnight.

146 Duplicate soil samples were collected from 4 of the 6 plots in July, 2013, when
147 the plants had reached late stem extension (Feekes growth stage 9, Zadoks growth stage
148 39; Large, 1954; Zadoks et al., 1974) corresponding to the period of maximum plant N
149 demand (AHDB, 2015). To estimate root density, intact soil cores were taken to a depth
150 of 80 cm using a Cobra-TT percussion hammer corer (Eijkelkamp Agrisearch
151 Equipment, 6987 EM Giesbeek, The Netherlands). After removal from the soil, the
152 intact cores were split into 10 cm sections, the samples transferred to CO₂ permeable
153 polythene bags and placed at 4°C to await root recovery and soil analysis. As there were
154 very few roots in the 60-80 cm layer, soils were only analyzed to 60 cm for the microbial
155 N cycling and N pool size estimates. For root analysis, one of the duplicate cores was
156 maintained intact, however, for the remaining soil analyses, the second soil core was
157 sieved to pass 2 mm, removing any vegetation, stones and earthworms and experiments
158 started within 48 h of field collection.

159

160 2.2. Quantification of root length density and soil respiration

161 Roots were washed from the soil cores by a combination of mechanical shaking
162 and flotation using a 1 mm mesh to capture roots. The roots were then placed on 20 ×
163 20 cm clear plastic plates and root length determined with WinRhizo[®] (Regent
164 Instruments Inc., Ville de Québec, Canada).

165 Basal respiration was determined on field-moist soil (50 cm³) in the laboratory at
166 20°C over 24 h using an SR1 automated multichannel soil respirometer (PP Systems
167 Ltd, Hitchin, UK). Visible roots were removed prior to analysis. The mean respiration
168 rate was determined for the last 6 h of the measurement period when the CO₂ efflux
169 rates had quasi-stabilized.

170

171 2.3. Soil solution extraction and soil chemical analysis

172 Soil N availability was estimated according to Jones and Willett (2006). Briefly,
173 5 g of field-moist soil was extracted with 25 ml of 0.5 M K₂SO₄ on a reciprocating
174 shaker (Edmund Bühler GmbH, SM-30, Germany; 200 rev min⁻¹) for 60 min. After
175 shaking, samples were centrifuged (10 min; 1699 g) and the supernatant recovered and
176 stored at -20°C to await analysis.

177 Soil solution samples were analyzed for dissolved organic C and total dissolved
178 N (TDN) using a Multi N/C 21005 (Analytik-Jena AG, Jena, Germany). Total amino
179 acid-N was determined fluorometrically using the *o*-phthaldialdehyde-β-
180 mercaptoethanol procedure of Jones et al. (2002). Nitrate and NH₄⁺ were analyzed
181 colorimetrically using the methods of Miranda et al. (2001) and Mulvaney (1996)
182 respectively. Dissolved organic N (DON) was calculated by subtraction of inorganic N
183 (NO₃⁻ and NH₄⁺) from TDN.

184 Total C and N of soils were determined on ground soil using a Truspec CN
185 analyzer (Leco Corp., St Joseph, MI, USA). Soil pH and electrical conductivity (EC)
186 were determined in soil:distilled water extracts (1:5 v/v) with standard electrodes, while
187 moisture content was determined by oven drying (105°C, 24 h). The gravimetric
188 moisture contents were corrected for stone-corrected bulk density to allow expression
189 of water content on a volumetric basis.

190

191 2.4. Net N mineralization and nitrification

192 Net N mineralization was determined by anaerobic incubation according to
193 Waring and Bremner (1964) and Kresoivć et al. (2005). Briefly, 10 g of field-moist soil
194 was placed in 50 cm³ polypropylene tubes and anaerobic conditions imposed by filling
195 the tubes with distilled water and then sealing the tubes. Soil samples were then
196 incubated for 7 d in the dark at 40°C. Subsequently, solid KCl was added to achieve a
197 final concentration of 1 M KCl and the samples extracted by shaking for 60 min (200

198 rev min⁻¹). The extracts were then centrifuged (1699 g, 10 min) and NH₄⁺ determined
199 as described previously. Net ammonification was calculated as the amount of NH₄⁺
200 present after 7 d minus that present at the start of the incubation.

201 Net nitrification was determined according to Hart et al. (1994). Briefly, 5 g of
202 field-moist soil from each soil layer was placed in a 50 cm³ polypropylene tube. The
203 tubes were then loosely sealed and the samples incubated in the dark at 20°C. After 30
204 d, the soil was subsequently extracted with 0.5 M K₂SO₄ and NO₃⁻ and NH₄⁺ determined
205 as described above. Net ammonification and nitrification was calculated as the amount
206 of NO₃⁻ and NH₄⁺ present after 30 d minus that present at the start of the incubation.

207

208 *2.5. Amino acid, peptide, protein and glucose turnover*

209 To estimate rates of DON turnover, the mineralization of amino acids,
210 oligopeptides and protein were determined. For comparison, the turnover of glucose was
211 also used as a general reporter of soil microbial activity (Coody et al., 1986). Briefly,
212 field-moist soil (5 g) was placed in 50 cm³ polypropylene containers and 0.5 ml of either
213 ¹⁴C-labelled glucose (25 mM, 1.85 kBq ml⁻¹), amino acids (10 mM, 1.55 kBq ml⁻¹),
214 peptides (25 mM, 1 kBq ml⁻¹) or protein (13.2 mg l⁻¹, 51 kBq ml⁻¹) added to the soil
215 surface (Farrell et al., 2011). After the addition of each ¹⁴C-substrate to the soil, a ¹⁴CO₂
216 trap containing 1 ml of 1 M NaOH was placed above the soil and the tubes sealed. With
217 the exception of protein, the tubes were then incubated at 20°C for 30 min after which
218 the NaOH traps were removed to determine the amount of substrate mineralized. In the
219 case of protein, the procedure was identical except that the incubation period was 24 h.
220 The ¹⁴C content of the NaOH traps was determined with Wallac 1404 liquid scintillation
221 counter (Wallac EG&G, Milton Keynes, UK) after mixing with Scintisafe3 scintillation
222 cocktail (Fisher Scientific, Loughborough, UK). The amino acids consisted of an
223 equimolar mix of 20 different L-amino acids (L-glycine, L-isoleucine, L-arginine, L-

224 glutamine, L-phenylalanine, L-histidine, L-asparagine, L-valine, L-threonine, L-leucine,
225 L-alanine, L-methionine, L-cysteine, L-lysine, L-tryptophan, L-serine, L-proline, L-
226 glutamate, L-aspartate acid, L-ornithine) while the L-peptides consisted of a mixture of
227 equimolar L-dialanine and L-trialanine. The mixed soluble plant protein was purified
228 from ^{14}C -labelled tobacco leaves (American Radiolabeled Chemicals Inc., St Louis,
229 MO, USA).

230 To determine the rate of arginine mineralization, 0.5 ml of a ^{14}C -labelled L-
231 arginine solution (25 mM; 2.17 kBq ml $^{-1}$; Amersham Biosciences UK Ltd, Chalfont St
232 Giles, Bucks, UK) was added to 5 g of field-moist soil and the rate of $^{14}\text{CO}_2$ evolution
233 measured over a 48 h as described in Kemmitt et al. (2006). After 48 h, the net amount
234 of NH_4^+ and NO_3^- produced from the added arginine was determined by extracting the
235 soil with 25 ml 0.5 M K_2SO_4 and subsequent analysis as described previously.

236

237 2.6. Mineralization of plant-derived C

238 The microbial turnover of complex, plant-derived C across the different soil
239 depths was evaluated according to Glanville et al. (2012). Briefly, high molecular
240 weight (MW) plant material was prepared by heating 2.5 g of ^{14}C -labeled *Lolium*
241 *perenne* L. shoots (Hill et al., 2007) in distilled water (25 ml, 80°C) for 2 h. The extract
242 was then centrifuged (1118 g, 5 min) and the soluble fraction removed. The pellet was
243 then resuspended in distilled water and the heating and washing procedure repeated
244 twice more until >95% of the water soluble fraction had been removed. The pellet
245 remaining was dried overnight at 80°C and ground to a fine powder.

246 The mineralization dynamics of the high MW plant material was determined by
247 mixing 100 mg of ^{14}C -labelled plant material with 5 g of field-moist soil. The production
248 of $^{14}\text{CO}_2$ was monitored as described above for the low MW substrates but over 40 d.
249 To ensure that water was not limiting, the experiment was also repeated but after the

simultaneous addition of distilled water (to reach field capacity) and the ^{14}C -labelled plant material.

2.7. Nucleic acid extraction and quantitative PCR (qPCR)

For each soil sample, DNA was extracted from duplicate 800 mg sub-samples using UltraClean[®] DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Cell lysis was performed using a Mini Bead beater (BioSpec Products Inc., Bartlesville, OK) at 2500 rev min⁻¹ for 2 min. Duplicate DNA extractions were combined to give a total extract volume of 100 μl .

Functional genes, archaeal and bacterial *amoA*, were quantified using a ViiA7 qPCR machine (Thermo Fisher Scientific, Scoresby, Australia). Each 20 μl qPCR reaction contained 10 μl of Power SYBR[®] Green PCR Master Mix (Thermo Fisher Scientific). 0.2 μl of the specific forward and reverse primer at a concentration of 10 μM , 2 μl BSA (Ambion UltraPure BSA; 5 mg ml⁻¹; Thermo Fisher Scientific), 8 ng template DNA and sterile water to 20 μl . Primers and thermal cycling conditions for both bacterial (primers amoA-1F and amoA-2R) and archaeal (primers Arch-amoAF and Arch-amoAR) *amoA* genes were as described previously (Banning et al., 2015). Melting curves were generated for each qPCR run and fluorescence data was collected at 78°C to verify product specificity. Each qPCR reaction was run in triplicate. Standard curves were generated using dilutions of linearized cloned plasmids. Template amplified with each primer pair described above, was cloned with the P-GEM T-easy system (Promega Inc., Madison, WI), plasmid DNA extracted and inserts sequenced using Big Dye Terminator chemistry (Australian Genome Research Facility, Western Australia) to confirm correct length and identity. The standard curve gene sequences were as described previously (Barton et al., 2013). Standard curves generated in each reaction

275 were linear over four orders of magnitude (10^4 to 10^7 gene copies) with r^2 values greater
276 than 0.99. Efficiencies for all quantification reactions were 80-100%.

277

278 2.8. *Microbial community structure*

279 Microbial community structure was measured by phospholipid fatty acid
280 (PLFA) analysis following the method of Buyer and Sasser (2012). Briefly, samples (2
281 g) were freeze-dried and Bligh-Dyer extractant (4.0 ml) containing an internal standard
282 added. Tubes were sonicated in an ultrasonic bath for 10 min at room temperature before
283 rotating end-over-end for 2 h. After centrifuging (10 min) the liquid phase was
284 transferred to clean 13 mm \times 100 mm screw-cap test tubes and 1.0 ml each of
285 chloroform and water added. The upper phase was removed by aspiration and discarded
286 while the lower phase, containing the extracted lipids, was evaporated at 30°C. Lipid
287 classes were separated by solid phase extraction (SPE) using a 96-well SPE plate
288 containing 50 mg of silica per well (Phenomenex, Torrance, CA). Phospholipids were
289 eluted with 0.5 ml of 5:5:1 methanol:chloroform:H₂O (Findlay, 2004) into glass vials,
290 the solution evaporated (70°C, 30 min). Transesterification reagent (0.2 ml) was added
291 to each vial, the vials sealed and incubated (37°C, 15 min). Acetic acid (0.075 M) and
292 chloroform (0.4 ml each) were added. The chloroform was evaporated just to dryness
293 and the samples dissolved in hexane. The samples were analyzed with a 6890 gas
294 chromatograph (Agilent Technologies, Wilmington, DE) equipped with autosampler,
295 split-splitless inlet, and flame ionization detector. Fatty acid methyl esters were
296 separated on an Agilent Ultra 2 column, 25 m long \times 0.2 mm internal diameter \times 0.33
297 μ m film thickness. Standard nomenclature was followed for fatty acids (Frostegård et
298 al., 1993).

299

300 2.9. *Statistical and data analysis*

301 Statistical analysis of the results was carried out by ANOVA followed by Tukey
302 HSD post hoc test and linear regression using SPSS v14 (IBM UK Ltd, Hampshire, UK)
303 with $P < 0.05$ used as the level to define significance. Analysis of differences in qPCR
304 abundances of bacterial and archaeal *amoA* across soil depth was performed by analysis
305 of variance (one-way ANOVA) using GenStat (15th edition; Lawes Trust, Harpenden,
306 UK). Principal component analysis was performed in R’.

307

308 **3. Results**

309 *3.1. Crop and soil characteristics*

310 As expected, crop height showed a sigmoidal extension pattern over the growing
311 season with full stem extension evident after 8 weeks (Fig. 1a). Crop biomass also
312 showed a sigmoidal growth pattern, however, above-ground biomass continued to
313 increase up until week 13 due to progressive grain filling (Fig. 1a).

314 Corresponding with the period of maximum crop development and low rainfall,
315 soil water content declined dramatically between weeks 5-8 in both the topsoil and
316 subsoil; with soil water in the subsoil being consistently lower than in the topsoil ($P <$
317 0.05 ; Fig.1b). At week 9, significant amounts of rainfall caused recharge of the soil
318 profile with the topsoil retaining significantly more water than the subsoil ($P < 0.01$;
319 Fig. 1b).

320 Root length density decreased down the soil profile, with the vast majority
321 located in the topsoil (Fig. 2a). Less than 4% of total root length density was in the
322 subsoil below 30 cm. Soil total and bio-available C pools also decreased with increasing
323 depth (Table 1). Soil basal respiration was significantly greater ($P < 0.05$) in the 0-20
324 cm layer (Fig. 2b) with the pattern matching that of root density distribution.

325

326 *3.2. Mineral N cycling*

Ammonium and nitrate concentrations in the field-collected samples were significantly greater in the surface (0-10 cm) layer ($P < 0.05$) than in the deeper soil horizons (Table 1). Overall, the patterns of N mineralization in the aerobic and anaerobic incubations were similar, decreasing in an exponential pattern down the soil profile (Fig. 3). The concentration of NH_4^+ after 30 d of aerobic incubation only increased significantly in the 10-20 cm soil layer (Fig. 3). Aerobic net N mineralization within the 0-20 cm layer of the soil profile was significantly greater ($P < 0.05$) compared to the 40-60 cm layer (Fig. 3). In contrast to the aerobic incubation, the anaerobically incubated soils showed large increases in NH_4^+ concentration at all depths, with the largest increase occurring in the surface soil layer (Fig. 3).

3.3. Low molecular weight carbon substrate mineralization

Mineralization rates of low molecular weight C molecules tended to decrease slightly with depth (Fig. 4). While substrate mineralization in the topsoil (0-30 cm) was significantly greater compared to the subsoil there was still considerable mineralization occurring at 50-60 cm (Fig. 4). There was a 10,000 fold difference between protein and amino acid mineralization rates, with rates in the order amino acid > peptide > glucose > protein.

3.4. Arginine and plant residue turnover

The initial (0-6 h) arginine C mineralization rate decreased with soil depth ($P < 0.05$) (Fig. 5a). However, by 48 h the amount of arginine mineralization was statistically similar at all soil depths. While the rate of mineralization was linear in the topsoil, however, a lag phase in mineralization was observed in the subsoil horizons (data not shown). The net amount of NH_4^+ produced from the added arginine significantly increased with soil depth (Table 2). In contrast, however, the net amount of NO_3^-

353 decreased significantly with increasing soil depth. Overall, the ratio of C mineralization
354 to N immobilization was greater in the topsoil than in the subsoil.

355 The rate of ^{14}C -labelled plant residue mineralization was much slower than those
356 of the simple C substrates. Notably there was no significant difference in turnover rates
357 between soil depths ($P > 0.05$; Fig. 5b).

358

359 3.5. AOA and AOB gene abundances

360 Nitrification capacity, as assessed by *amoA* gene abundance, was present
361 throughout the soil profile. At every soil depth AOA gene abundance was significantly
362 lower ($P < 0.01$) than AOB (Fig. 6). For AOB *amoA* gene copies ranged from 1×10^7
363 to $2 \times 10^8 \text{ g}^{-1}$ dry soil while AOA *amoA* gene copies ranged from 2 to $5 \times 10^5 \text{ g}^{-1}$ dry
364 soil. There was no significant effect of depth on AOA population abundance ($P > 0.05$)
365 but there was a significant effect of depth on AOB population abundance ($P < 0.05$)
366 whereby AOB gene abundance was significantly lower ($P < 0.05$) in the subsoil below
367 30 cm than in topsoil (Fig. 6).

368

369 3.6. Microbial community structure

370 Total PLFA significantly decreased below 30 cm depth; with the amount of total
371 PLFA relatively constant within topsoil and subsoil layers (Fig. 7a). Overall, the relative
372 proportion of major microbial groups was quite similar at the different soil depths. The
373 proportion of fungi and actinomycetes significantly increased with soil depth ($P < 0.01$
374 and 0.001 respectively) while the relative abundance of Gram-positive and Gram-
375 negative bacteria both reduced ($P < 0.01$). The relative abundance of putative arbuscular
376 mycorrhizal PLFAs (16:1 w5c) was similar at all depths (data not presented). Principal
377 component analysis of the PLFA data revealed a separation of the topsoil and subsoil
378 microbial communities (Fig. 8).

379

380 **4. Discussion**

381 *4.1. Changes in microbial biomass, activity and community structure with depth*

382 As expected, root abundance, microbial biomass and basal respiration all
383 declined with soil depth (Kramer et al., 2013; Li et al., 2014; Loeppmann et al., 2016).
384 In many cases, these changes can be attributable to excess acidity and toxic levels of
385 Al^{3+} in the subsoil (Tang et al., 2011). In our study, however, soil pH did not vary down
386 the profile and therefore this does not represent a confounding factor. Microbial
387 community structure also shifted down the soil profile with the fungal-to-bacterial ratio
388 increasing with depth, presumably due to increased C limitation and the lower N
389 requirement of fungi rather than due to a shift in soil pH. This is in agreement with the
390 results of Sanaullah et al. (2016) in grasslands but contrasts with the results of Kramer
391 et al. (2013) and Stone et al. (2014) who showed either no effect or a strong decrease in
392 fungal-to-bacterial ratio with depth. Based on the slow growth of Gram+ bacteria and
393 their greater ability to survive C starvation (De Vries and Shade, 2013), we expected to
394 see an increased Gram+-to-Gram- ratio with depth. Although our results do support this
395 to some extent, the overall effect was quite small. Despite the low microbial biomass,
396 however, we demonstrate that high rates of both soluble and insoluble C and N turnover
397 can occur at depth. Generally, however, microbial processes have a tendency to be
398 greatest in surface layers, especially when soil disturbance is minimised (Murphy et al.,
399 1998). Numerous factors could contribute to lower microbial activity in deep soils. The
400 results obtained here show a much lower abundance of roots at depth so it is likely that
401 there is less soluble organic C or fresh particulate C being delivered to the subsoil via
402 root exudation and root/mycorrhizal turnover (Fontaine et al., 2007). The lack of
403 earthworm presence in our soil also prevents the bioturbation-driven delivery of C to
404 the subsoil and limits subsoil biological hotspots in the form of deep vertical earthworm

405 burrows (Uksa et al., 2015; Hoang et al., 2016). Microbes at depth therefore experience
406 strong C limitation which is supported by the decrease in C-to-N ratio with depth in
407 some soils (Rumpel and Kögel-Knabner, 2011). In addition, the increasing DOC-to-
408 DON ratio with depth suggests that the DOC may be becoming more chemically
409 recalcitrant (i.e. humic-like) down the soil profile. Other mechanisms which may also
410 restrict microbial activity in subsoils include: (1) an increased bulk density which may
411 suppress root growth; (2) greater structural aggregation which may both restrict root
412 access and promote the physical protection of C; (3) a greater abundance of clay and
413 oxyhydroxides which may stimulate the chemical protection of C; and (4) greater
414 moisture contents and resulting anoxia which may suppress root and microbial activity
415 (Kinyangi et al., 2006; Rumpel and Kögel-Knabner, 2011). In the context of our well
416 drained, sandy-textured soil we expect the influence of these factors to be relatively low.

417 Soluble organic N concentrations decreased with soil depth suggesting that the
418 microbial community could also be N limited at depth. Based on the evidence presented,
419 we ascribe these low concentrations to the low rate of DON supply from rhizodeposition
420 and SOM turnover combined with the rapid microbial removal of labile DON from
421 solution. As added soluble-N was readily mineralized to NH_4^+ in our subsoils we
422 conclude that subsoil microbial activity is driven more by C limitation rather than by N
423 limitation. This view is also supported by Jones et al. (2005) who demonstrated that the
424 microbial use of DON compounds was largely insensitive to N fertilizer regime and
425 more related to C availability than N availability in a range of agricultural soils. In
426 addition, we observed a relatively high concentration of NO_3^- at depth. NO_3^- tends only
427 to be utilized in large amounts by microorganisms under severe N deficiency due to the
428 energetic costs associated with its assimilation (in comparison to DON and NH_4^+), again
429 suggesting that the subsoil microbial community is not N limited (Abaas et al., 2012).

430

431 4.2. *Implications for subsoil C storage*

432 Reduced microbial activity at depth has led to the suggestion that subsoils may
433 have the potential to lock up additional C and that this could help offset
434 anthropogenically derived greenhouse gas emissions (Lynch and Wojciechowski, 2015;
435 Pierret et al., 2016; Gocke et al., 2017; Torres-Sallan et al., 2017). One widely proposed
436 mechanism to stimulate this transfer of C into subsoils is the use of crops with deep
437 rooting traits (Lavania and Lavania, 2009; Kell, 2011) or shifts towards less intensive
438 land management systems (Ward et al., 2016). It should be noted, however, that much
439 controversy surrounds the stability of C in subsoils with many reports suggesting it
440 persists for long time periods and is more stable than C in topsoils (Kramer and Gleixner,
441 2008; Müller et al., 2016). The evidence presented here clearly showed that while the
442 rates of plant residue turnover were initially slower in subsoils in comparison to topsoils
443 (0-24 h), these differences disappeared over longer incubation times (e.g. 30 d). This
444 suggests that the subsoil microbial community quickly adapted to an increased C supply.
445 This directly challenges the assumption that increasing the rate of C supply to subsoils
446 will lead to greater long term C storage. It is also consistent with measurements showing
447 that most subsoil C is of recent origin and not very stable (Hobley et al., 2017; Zhang et
448 al., 2014). Our results also support the results from Brauer et al. (2013) and Matus et al.
449 (2014) who suggest that C storage in subsoils is mainly driven by microbially-processed
450 C being translocated down the soil profile as DOC and then becoming chemically
451 protected, rather than C generated in situ within subsoils.

452 It should be emphasized that the discussion above mainly relates to the potential
453 for accumulating subsoil C over a limited number of cropping cycles (i.e. 1-10 y). Over
454 longer time scales it is conceivable that small amounts of C may become progressively
455 stabilized in subsoils leading to substantial C increases over decadal time scales. Current
456 evidence suggests that long-term shifts in agronomic management (>40 years) targeted

at surface residue management and tillage regime can substantially increase topsoil C levels, but that they have limited capacity to alter subsoil C storage (Jarvis et al., 2017; Kinoshita et al., 2017). This provides strong evidence that C migration from top- to subsoils is not an effective mechanism for promoting C storage in deeper soil layers. An alternative to relying on roots to deliver C to subsoils is the deep incorporation (>50 cm depth) of crop residues into soil (Alcantara et al., 2017; Cui et al., 2017). Unlike cereal roots whose C-to-N ratio ranges from 15-30, the low N content of crop residues (C-to-N ratio = 50-80) is more likely to favour C retention (and may additionally suppress N losses via leaching). It is clear, however, that more long-term field trials are required to critically address whether deeper rooting crops lead to enhanced C sequestration.

467

4.3. Variation in soil N cycling with depth

Protein represents the major input of organic N into cropping soil systems. Therefore, the mineralization of protein, oligopeptides and amino acids is an important part of the N cycle and supplies the substrate for inorganic N production and therefore root N uptake (Jones et al., 2013). In topsoils it has been proposed that the breakdown of proteins to peptides is the main rate limiting step in the soil N cycle (Jan et al., 2009) and the evidence presented here clearly suggests that this is also the case for subsoils. However, when expressed per unit of microbial biomass, protein breakdown rate was much greater in subsoils than topsoils. Contrary to Loeppmann et al. (2016), this could either imply that the proteases have a greater substrate affinity at depth or it may relate to greater substrate availability (i.e. less substrate sorption to the solid phase). More work is therefore required to understand the factors regulating the production and behaviour of proteases in subsoils. Pinggera et al. (2015) recently demonstrated that subsoil protease activity was upregulated when abundant substrate was available but was repressed if sufficient inorganic N was present. In line with our results, this suggests

483 that the microbial community will readily respond to substrate addition. Further, it also
484 suggests that addition of high C:N residues (e.g. values >20) might stimulate positive
485 priming and the mining of subsoil SOM to release N.

486 Arginine addition caused the rapid mineralization of amino acid-N to NH_4^+ at all
487 soil depths, again demonstrating that ammonification was not a rate limiting step at any
488 point in our soil profile. In addition, our soil incubation results showed that NH_4^+ only
489 increased under anaerobic conditions, when C degradation and nitrification are oxygen
490 limited. As rapid ammonium oxidation occurred readily under aerobic conditions it also
491 supports the premise that N cycling in both topsoils and subsoils is limited by upstream
492 elements in the N cycle (i.e. substrate availability for protease action).

493 Our results also reveal much greater nitrification potential in surface soils than
494 at depth which may be indicative of a larger active community of nitrifiers. Therefore,
495 more NH_4^+ would be transformed to NO_3^- in the surface soil and potentially more could
496 be lost as N_2O or N_2 . We show that the vertical distribution of both AOA and AOB were
497 strongly correlated with each other ($r^2 = 0.92$, $P < 0.01$) and also with total microbial
498 PLFA ($r^2 > 0.82$, $P < 0.05$). Further, AOB abundance closely correlated with the wider
499 Gram- bacterial community of which it forms part ($r^2 = 0.87$, $P < 0.05$). This implies that
500 nitrification has no specialist niche in the soil profile relative to the more general aspects
501 of soil organic C and N cycling. The results also do not support the proposal that AOB
502 and AOA communities behave differently in different soil layers (Wang et al., 2014). In
503 surface soils we found an increased abundance of AOB when compared to lower depths,
504 and additionally report that AOA abundance was much lower than for AOB (ca. 200-
505 fold) and did not vary as greatly with depth. Our results contrast with Fisher et al. (2013),
506 Uksa et al. (2014), Wang et al. (2014) and Liu et al. (2016) who all showed that AOA
507 abundance was much greater than AOB, particularly in subsoils. From our results, we
508 infer that AOB are likely driving nitrification in this system due to the increase in

509 measured nitrification in conjunction with an increase in AOB but not AOA abundance
510 in surface soils. It is also likely that AOB are more active and can respond more quickly
511 to additions of organic N and NH_4^+ derived from this (Di et al., 2010). This is consistent
512 with a number of other studies (Di et al., 2009, 2010; Barton et al., 2013; Banning et al.,
513 2015) who also suggest that AOB are likely driving topsoil nitrification.

514 In addition, we found that NH_4^+ concentrations in the field were significantly
515 greater in the topsoil than at depth, which we ascribe to its higher organic matter and
516 cation exchange capacity. This likely favours AOB over AOA with previous studies
517 showing that AOA may only have a competitive advantage at low ammonium
518 concentrations due to their greater substrate affinity (Martens-Habbena et al., 2009) or
519 due to greater sensitivity to growth inhibition at high ammonium concentrations (Prosser
520 et al., 2012). Soil pH is often described as having a significant influence on AOA and
521 AOB abundance, although reports are not consistent. Some studies have reported AOB
522 to be more sensitive than AOA to pH changes (Nicol et al., 2008; Yao et al., 2011). For
523 example Yao et al. (2011) observed that AOB were more abundant in neutral and
524 alkaline conditions than in acidic conditions, whereas there was no correlation between
525 pH and AOA abundance. In contrast, Pereira e Silva et al. (2012) found soil pH did not
526 influence AOB abundance but did increase AOA abundance; while Nicol et al. (2008)
527 reported only AOA abundance and not AOB, was influenced by pH. In the current study
528 pH did not change with depth and thus the increased abundance of AOB in the surface
529 is not likely related to pH in this study.

530 It should also be noted that although there may be a significant reserve of
531 nutrients at depth, these may be also physically or chemically protected, especially in
532 well structured subsoils. It is therefore important for future studies to consider not only
533 the size of the nutrient pool, but also the gross flux through this pool and its bio-
534 accessibility

535

536 *4.4. Implications of N cycle variations with depth for root uptake*

537 Deeper rooting may promote the more efficient use of nutrients such as N and P
538 (Lynch and Wojciechowski, 2015). However, we hypothesized that roots at the surface
539 would be involved more in nutrient uptake than those at depth. The greater root length
540 observed in this study corresponds with the areas of greater microbial activity, N
541 concentrations and turnover rates. Therefore, it is likely that much more N is taken up
542 by surface roots than those at depth. The surface soil is also likely to be the area where
543 microbial N demand is greatest. Greater root length in topsoils would therefore allow
544 for greater competition with microbes. The greater bulk density at depth may also
545 suppress root growth making the access of nutrients more difficult (Salome et al., 2010).

546 Water uptake is as important a function of plant roots as nutrient uptake. The
547 uptake rate of water is often proportional to root length density (Hinsinger et al., 2009;
548 Hodge et al., 2009). During drought, soil surfaces dry, limiting both water and,
549 potentially, nutrient uptake in roots near the surface. This can lead to near-surface roots
550 dying and greater root growth at depth (Smucker et al., 1991). In dry conditions, deeper
551 roots could become vital for maintaining plant nutrient uptake. In addition, more roots
552 at depth could lead to a greater input of exudates which would increase microbial
553 activity and decrease nutrient loss (Fisk et al., 2015). It may also promote the microbial
554 priming of subsoil SOM and the loss of stable C from soil (Fontaine et al., 2007).

555 Contrary to expectation, the subsoil appeared to retain less water than the topsoil.
556 We ascribe this to its lower SOM content which is known to aid water retention and
557 promote soil structure (Rawls et al., 2003). Further, the subsoil dried out and rewet at a
558 similar rate to the topsoil. This does not support the hypothesis that soil moisture
559 becomes proportionally more available in subsoil as the soil progressively dries out due
560 to evapotranspiration losses. Our results suggest that irrespective of root length density,

561 water is removed evenly throughout the soil profile to balance plant demand, or less
562 likely, that plant-mediated hydraulic lift is redistributing water from deeper soil layers
563 to the surface. This suggests that drying out of the soil profile does not induce spatial
564 niche partitioning in N availability.

565

566 *4.5. Conclusions*

567 In terms of plant-microbial nutrient cycling, subsoils remain understudied in
568 comparison to topsoils. In addition to providing water to plants, however, recent reviews
569 have suggested that subsoils may represent an important store of nutrients and have the
570 potential to sequester large amounts of C (Torres-Sallan et al., 2017). Consequently,
571 there is a growing view that subsoils should be actively managed to optimise their
572 functioning (e.g. by mechanical or plant-based interventions; Kell et al., 2011; Tang et
573 al., 2011; Alcantara et al., 2016, 2017). The results presented here suggest that although
574 the subsoil has a low and slightly different microbial community than the topsoil, in
575 terms of C cycling, the subsoil microbial community rapidly responds to new inputs of
576 organic C and N. This suggests that the use of deeper rooting plants may not enhance
577 long-term C storage in subsoils, especially if they destabilize subsoil SOM through
578 rhizosphere priming. Our results also show that, as expected, root proliferation is
579 greatest in the region of the soil profile where nutrient cycling is greatest. At present,
580 the routine sampling of agricultural subsoils is costly and problematic. Further, subsoils
581 can be expected to have higher spatial heterogeneity than topsoils. Combined, this
582 makes it difficult to make informed decisions for active subsoil management. We
583 conclude that the potential future importance of subsoils in sustainable agriculture may
584 have been overstated.

585

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591

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871 **List of Figure Captions**

872 Fig. 1. Crop biomass and stem extension (Panel A) and soil water content in the topsoil
873 (0-30 cm) and subsoil (30-60 cm) layers (Panel B) during the wheat cropping
874 cycle from planting to harvest. Values represent mean \pm SEM ($n = 6$). * and ** in
875 Panel B indicate significant differences between depths at the $P < 0.05$ level and
876 $P < 0.01$ level respectively. Note: Soil profiles for biochemical and molecular
877 analysis were collected 8 weeks after planting.

878 Fig. 2. Density of primary (first order) and lateral (second and third order) roots (Panel
879 A) and basal soil respiration at different depths in an agricultural wheat cropping
880 soil (Panel B). Values are means \pm SEM ($n = 5$). All measurements were made 8
881 weeks after planting. Different letters indicate significant differences between
882 depths at the $P < 0.05$ level (Tukey's HSD).

883 Fig. 3. Net ammonification after incubation under anaerobic conditions for 7 days or net
884 mineralization (ammonification and nitrification) after incubation under aerobic
885 conditions for 30 days at different soil depths in an agricultural wheat cropping
886 soil. Values represent means \pm SEM ($n = 4$). Different letters indicate significant
887 differences between depths at the $P < 0.05$ level (Tukey's HSD; lowercase for
888 anaerobic incubation and uppercase for aerobic incubation).

889 Fig. 4. Mineralization of ^{14}C -labelled glucose, amino acids, oligopeptides and protein at
890 different soil depths in an agricultural wheat cropping soil. Values represent
891 means \pm SEM ($n = 4$). Different letters indicate significant differences between
892 depths at the $P < 0.05$ level (Tukey's HSD).

893 Fig. 5. Cumulative percentage of ^{14}C - arginine mineralization (Panel A) and cumulative
894 percentage of ^{14}C -*Lolium perenne* shoots mineralization (Panel B) at different soil
895 depths in an agricultural wheat cropping soil. Values represent means \pm SEM ($n =$
896 4).

897 Fig. 6. Bacterial (AOB) and archaeal (AOA) *amoA* gene copy numbers at different soil
898 depths in an agricultural wheat cropping soil. Values are means \pm SEM ($n = 4$).
899 Different letters indicate significant differences between depths at the $P < 0.05$
900 level (Tukey's HSD).

901 Fig. 7. Total microbial PLFA (Panel A) and the relative abundance of specific microbial
902 PLFA markers (Panel B) at different soil depths in an agricultural wheat cropping
903 soil. Values are means \pm SEM ($n = 4$). In Panel B the fungal PLFA marker data
904 have been multiplied $\times 10$ for scaling purposes. Different letters indicate
905 significant differences between depths at the $P < 0.05$ level (Tukey's HSD).

906 Fig. 8. Principal component analysis for PLFAs (taxonomic groups based on PLFAs) as
907 a function of soil depth. Two scales are used, the ± 3.0 scale refers to the loadings
908 of the samples at different depths and the ± 1.0 scale refers to the loadings of the
909 different taxonomic groups (variables). The percent of variation is included on
910 each Principal Component (PC).